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Non-invasive Monitoring of Antioxidants and Stress in Captive Indian Leopards

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ABSTRACT

Background: Captive Indian leopards are exposed to different kind of stresses part of which can be alleviated through supplementation of carotenoids. However, invasive monitoring of antioxidants is often pro-act as a stressor itself. Hence, use of non-invasive monitoring of antioxidants and stress would be desirable. However, before recommendation, such non-invasive procedures must be compared with conventional ones. Hence, this experiment was designed to compare the cortisol and total antioxidants (TAA) status measured either in serum or faecal samples.

Methods: Three diets were tested on twelve adult leopards (7 M and 5 F, BW ranging from 45-63 kg) in an experiment based on replicated Latin square design comprising of three treatments, three periods and three sequences and four animals in each sequence. The ratio of buffalo meat: chicken carcass was 100:0, 90:10 and 80:10 in groups CON, GI and GII, respectively.

Result: Intake and absolute quantity of carotenoids absorbed increased (P<0.01) with increased level of chicken carcass; however, efficiency of absorption was lower (P<0.05) in GII as compared to other two groups. Faecal concentrations of cortisol decreased (P<0.0001) and TAA increased (P<0.0001) with increased level of carotenoids in the diet. Irrespective of the dietary treatments, concentration of cortisol was lower (P<0.0001) and TAA was higher in faecal as compared to serum samples. Regression analysis revealed positive relationship between serum and faecal sample assay with respect to both TAA and cortisol. Thus, faecal samples could be used to monitor cortisol and antioxidant status in Indian leopards. Assay of faecal samples indicated that replacement of buffalo meat with chicken at 20% in the diet improved the antioxidants and alleviated stress in captive leopards.

Key words: Broiler meat, Carabeef, Faecal cortisol, Non-invasive, Panther, Total antioxidant activity.

INTRODUCTION

Leopard (*Panthera pardus*) is a Near Threatened (NT) species, concerned with the declined population of leopards in the wild, zoo community is coming forward to make their contribution in *ex-situ/in-situ* conservation. However, to make such programme successful, it is mandatory that proper nutrition and diet be provided to the animals which will improve performance and welfare of the animals. Information available on feeding and nutrition of zoo-housed leopards are meagre which mostly pertains to intake and utilization of major nutrients (Pradhan *et al.,* 2015; Sarode *et al*., 2018, 2019; Durge *et al*., 2018). In free range, leopard is an opportunistic predator that preys on several species like sambar, spotted deer, barking deer, four-horned antelope, mouse deer, wild pig, gaur, hare, domestic cattle, and many other mammals and birds (Andheria *et al*., 2007). However, in captivity they are fed solely on buffalo meat. Feeding of monotonous diet in confinements may adversely influence animal welfare (Catanese *et al*., 2013). Increase in variety in the diet had earlier been reported to improve welfare in animals including felid (Sturgess and Hurley, 2007). Further, buffalo meat (BM) contains either no or very negligible amount of carotenoids (Sarode *et al*., 2019) as compared to natural preys of leopards that accumulate moderate to good amount of carotenoids (Slifka *et al*., 1999). In contrast, birds are good accumulator of carotenoids. Research conducted earlier demonstrated that replacement of 20% BM with chicken improved the concentration of carotenoids with an increase serum level of antioxidant enzymes (Sarode *et al*., 2019).

However, Invasive methods require capture and restraint that imposes stress to animals. Hence, non-invasive methods are encouraged in experiments with wild animals. It is generally accepted that plasma concentration of gluco-corticoids metabolites accord well with faecal concentrations of glucocorticoid metabolites (GCM). Serum and faecal

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cortisol responded in a similar manner to an injection of ACTH in cheetah (Terio *et al*., 1999) and feces is the predominant route of excretion of GCM in felids, 86% of the GCM were recovered in feces (Graham and Brown, 1996). Although relatively rare there are studies indicating that antioxidant activities in feces and faecal water can be used a marker of antioxidant status (Garsetti *et al*., 2000; Bianchi *et al*., 2010). Thus, it was of interest to see if the serum concentrations of cortisol and TAA are reflected in faecal concentration of these metabolites in captive Indian leopards or not. Hence, the present experiment was conducted to compare the cortisol and TAA status of zoo-housed Indian leopard measured either in serum or faecal samples.

MATERIALS AND METHODS

Study design

Twelve adult leopards (7 M and 5 F, age 3-14 years, body weight (BW) ranging from 45-63 kg) were randomly allocated into 3 groups of 4 each following replicated latin square design comprising of 3 treatments, 3 periods and 3 sequences (each sequence containing 4 animals). The ratio of BM: chicken was 100:0, 90:10 and 80:10 in groups CON, GII and GII, respectively. Each experimental period comprised of 21 days.

Feeding and housing management

The experimental protocol was approved by the Institute Animal Ethics Committee (IAEC) of ICAR-IVRI, Izatnagar 243 122, India and The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Ministry of Environment and Forest, Government of India. Details of their housing, feeding and health management were detailed in an earlier paper in which we had shown the benefits of replacing 20% of BM with chicken on intake, nutrient utilization and invasive monitoring of antioxidant profile in leopard (Sarode *et al*., 2019). Briefly, all animals were housed individually in enclosures having indoor (3×3×2 m) and outdoor (9×9×5 m) facilities. Fresh buffalo meat was fed @ 2.5-3 kg/d, 6 day a week, at 17:00 h. Drinking water was provisioned at all times.

Sampling and measurements

In order to determine the absorption efficiency of carotenoids, a digestion trial was conducted from days 17th to 20th of each period on each of the animals. During digestion trial, animals were housed individually inside their respective inner enclosure. Accurately measured amount of chicken and buffalo meat was officered to each of the leopards. An accurate record of refusals of buffalo meat and total volume of feces voided were also maintained. Freshly collected chicken, BM and leftovers were mechanically (hammering, mincing, pounding) blended into a uniform mixture. Representative aliquot was taken for the determination of dry matter (DM) in duplicate.

Blood was collected from all the animals by puncturing coccygeal vein of each of the leopards into Vacationer tubes (BD Vacationer, Franklin Lake, NJ, USA), After clotting, samples were centrifuged at 3000 rpm for 10 min and collected serum samples were frozen at -20°C till laboratory analysis.

Chemical analysis

Samples of chicken, BM and its leftovers, and faeces were dried at 70°C for 72 h to determine the dry matter (DM) content (AOAC, 2000; method 950.46). Total carotenoids (TC) content of chicken, BM and faecal samples were determined following modified methods of AOAC (1973). In the original method TC was measured as β-carotene (µg/g) and reading was taken at 450 nm in acetone extract. We measured TC as lutein (µg/g) and spectrophotometer reading was taken at 446 nm in acetone extract.

Faecal and serum cortisol and antioxidant assay

Faecal samples for cortisol assay were processed according to the method of Santymire *et al*. (2011). Faecal cortisol assay was performed using a commercial RIA kit (Immunotech, Prague, Czech Republic) following protocol provided by the manufacturer.

Faecal concentration of TAA was measured according to the method of Garsetti *et al*., (2000). Serum TAC was measured according to the method of Koracevic *et al*. (2001). Serum concentration of cortisol was assayed using Gamma coated TM cortisol ^{125}I-RIA kit (Immunotech, Prague, Czech Republic) following protocols provided by the manufacturer.

Statistical analysis

Carotenoids intake was regressed upon proportion of chicken in the diet. Faecal concentration of cortisol and TAA were regressed upon carotenoids. Data pertaining to faecal concentrations of cortisol and TAA were regressed on concentration of these attributes in serum samples. Analysis of variance (ANOVA; one-way for intake and absorption of carotenoids, two-way for cortisol and TAA) was done. Treatment means were differentiated (Tukey's test) and statistical significance was declared at $P<0.05$. All statistical analysis was done using SPSS 17.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Intake and absorption of carotenoids

Intake of total carotenoids increased ($P<0.001$) with increased level of chicken in the diet ($Y=0.119X+0.532$; $R^2=0.978$). Amounts of carotenoids excreted in feces and apparently absorbed were higher ($P< 0.001$) in GII, followed by GII and CON (Table 1). Even though the amount of carotenoids absorbed increased with increased level of carotenoids intake ($Y=0.238X+0.0.064$; $R^2=0.943$), efficiency of absorption of carotenoids was lower ($P< 0.001$) in GII as compared to other two groups. It is of interest to note that felids are good accumulators of carotenoids and maintain a higher circulatory concentration of carotenoids (Slifka *et al*., 1999). As carotenoids cannot be synthesized *de novo*, a higher circulatory concentration would imply that these molecules are absorbed from dietary sources. We had earlier

demonstrated that circulatory concentration of total carotenoids is positively related to dietary concentration of carotenoids (Sarode *et al*., 2019). Here, in this experiment we have quantitatively measured the concentration of carotenoids absorbed by zoo-housed leopards though an accurate measure of carotenoids intake and outgo in feces. Our results show that leopards can absorb considerable quantity of total carotenoids of chicken origin. Research conducted earlier has shown that domestic cats can absorb lutein (Kim *et al*., 2000). Thus, it seems that there is similarity between the two species with respect to absorption of lutein. Absolute amount of lutein absorbed by the leopards increased in a dose dependent manner. Considering the important roles of carotenoids as antioxidants (Sarode *et al*., 2019) and imuno-modulators (Kim *et al*., 2000) and in prevention of macular degenerative disease, it seems beneficial to increase the proportion of chicken in the diet of zoo-housed large felids fed solely on buffalo meat. The efficiency of absorption of carotenoids, however, was decreased at higher level of intake. As carotenoids are lipophilic in nature, absorption of carotenoids is influenced by dietary fat. Plasma concentration of β-carotene was higher on high-fat diet as compared to low-fat diet (Dimitrov *et al*., 1988). Chicken contained less fat as compared to buffalo meat. Thus, increasing the dietary proportion of chicken might have reduced the supply of fats at intestine resulting in lower efficiency of absorption of carotenoids.

Antioxidant activity

Faecal concentration of TAA increased (P<0.001) with increased dietary supply of carotenoid (Y=5.911X+20.51; R^2=0.864). Irrespective of the dietary treatments, concentration of TAA was higher in faecal (Table 2) as compared to serum samples (P<0.0001). Regression

Table 1: Effect of gradual replacement of buffalo meat with chicken on total carotenoids intake, faecal cortisol and faecal antioxidant activity leopards.

Parameters	Treatments†			SEM	P Value
	CON	GI	GII		
Weekly feed consumption (kg)					
Buffalo meat	15.00	13.60	11.10	0.12	<0.001
Chicken	0	01.74	03.42	0.05	<0.001
Total (as fed)	15.00	15.34	14.52	0.20	0.176
Dry matter	04.72	04.75	04.44	0.15	0.168
Intake and apparent absorption of total carotenoids (TC)					
TC Intake (µg/d)	502^{a}	1850^{b}	3160^{c}	30.0	<0.001
TC excreted (µg/d)	330^{a}	1310^{b}	2360^{c}	26.6	<0.001
TC Absorb (µg/d)	172^{a}	540^{b}	800^{c}	16.6	<0.001
% TC Absorb	33.9^{c}	29.1^{b}	25.3^{a}	1.06	<0.001

^{a,b,c}Mean bearing different superscripted letter differ significantly.

†Animals in group CON were fed solely on buffalo meat, whereas, 10 and 20% of buffalo meat was replaced with chicken in groups GI and GII, respectively.

TC, Total carotenoids.

Table 2: Effect of gradual replacement of buffalo meat with chicken on total carotenoids intake, faecal cortisol and faecal antioxidant activity in zoo-housed Indian leopard (*Panthera pardus fusca*).

Parameters	Sampling procedure†		P-value		
	Invasive	Non-invasive	Sampling	Treatment	S*T
Total antioxidant activity (mmol/kg of feces, mmol/l of serum)					
CON	1.68^{a}±0.02	23.10^{a}±0.69	0.001	0.001	0.005
GI	2.01^{b}±0.01	32.1^{b}±0.85			
GII	2.30^{c}±0.02	38.9^{c}±0.70			
Cortisol (ng/g)					
CON	401^{c}±10.4	342^{c}±3.36	0.001	0.001	0.001
GI	352^{b}±5.7	301^{b}±2.78			
GII	289^{a}±7.7	268^{a}±3			

^{a,b,c}Mean (±SE) with different superscript in a row differ significantly.

†Leopards in group CON were fed normal zoo diet of buffalo meat without any chicken, whereas, 10 and 20% of buffalo meat was replaced with chicken in groups GII and GII, respectively.

analysis revealed positive relationship between serum and faecal sample assay with respect to TAA (Fig 1).

Antioxidant activities are most extensively studied in RBC and other tissues and to a limited extent in serum in animals including felids (Weydert and Cullen, 2010). In contrast there are only few studies that report antioxidant activities in feces and faecal water (Garsetti *et al*., 2000). Faecal antioxidants are indicator of antioxidant status of colon (Bianchi *et al*., 2010). Results of this experiment show that the antioxidant activity of the hindgut increases with increased proportion of dietary chicken that could be linked to increased supply of dietary carotenoids. Additionally, efficiency of absorption of carotenoids decreased with increased level of chicken carcass in the diet that might have resulted in increased antioxidant activity of the feces. Positive relationship that we have observed between faecal concentrations of TAA and dietary carotenoids indicates that carotenoids act synergistically with endogenous antioxidant system and boost up antioxidant activity in hindgut.

In order to compare the TAA measured either through invasive or non-invasive methods, we compared the faecal TAA values with serum TAA values reported earlier from our laboratory (Sarode *et al*., 2019). Results showed that total antioxidant activity was 13.75 to 16.91 folds higher in feces as compared serum. This corroborates well with earlier findings that reports 19 times greater total antioxidants in feces as compared to plasma (Rice-Evans and Miller, 1994). It is to be noted that hindgut is equipped with its own antioxidant defense mechanism comprising of sulfated glycoproteins, uric acid, coproporphyrins and other bile pigments (Stocker *et al*. 1990). Higher antioxidant activity of feces could be attributed to the presence of these antioxidant substances in the colon. Irrespective of invasive or noninvasive methods, antioxidant activity increased with increased proportion of chicken carcass in the diet, and was positively related with carotenoids intake. Thus, the response of supplementation of exogenous antioxidants can be studied by using non-invasive method as well. It is further evident from the positive relationship that we observed between serum and faecal antioxidant activity. Considering that invasive methods of studying antioxidant status need capture and restrain of animals that imposes stress, it would be desirable to use the non-invasive methods for measuring antioxidant status in wild animals.

Cortisol

Faecal concentration of cortisol showed a negative ($P<0.001$) relationship ($Y=-27.72\ X+355.2$; $R^2=0.894$) with dietary concentration of carotenoids (Fig 2). Irrespective of the dietary treatments, concentration of cortisol was lower (Table 2) in faecal as compared to serum samples ($P<0.0001$). Regression analysis revealed positive relationship between serum and faecal sample assay with respect cortisol (Fig 1).

Assay of cortisol indicate about the stress levels in animals. Cortisol can be measured either in samples of

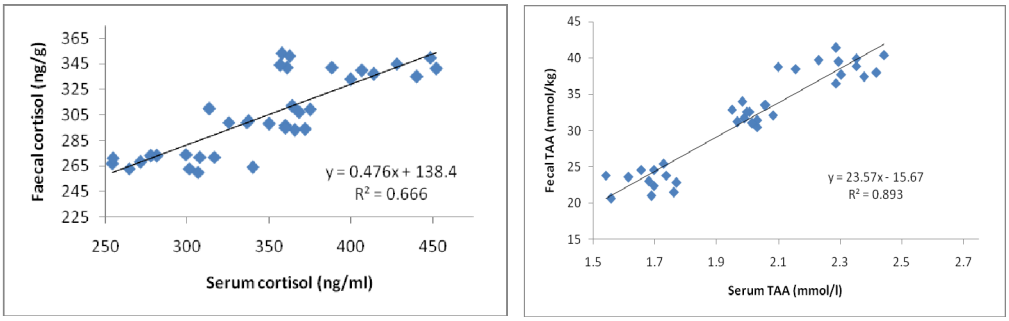

Fig 1: Regression of faecal cortisol and total antioxidant activity on serum cortisol and total antioxidant activity.

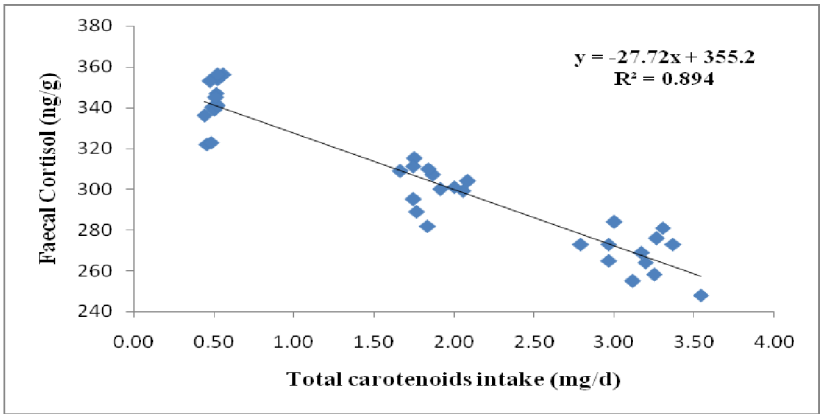

Fig 2: Regression of faecal cortisol on dietary carotenoids intake.

serum, saliva, or feces (Möstl and Palme, 2002). Serum concentration of cortisol can be affected rapidly with response to the stress induced by capture, restraint and sampling procedure that may confound the results. Additionally, glucocorticoids exhibit regular or episodic changes over time. Thus, measuring of cortisol over a short time frame could be misleading. These problems could be effectively overcome by measuring cortisol in faecal samples (Touma and Palme, 2005). In this experiment, faecal concentration of cortisol ranged from 248-356 ng/g. Considering that reference value of faecal cortisol in this species is not available, we compared the values obtained in this experiment with those reported in other felids. Faecal concentration of cortisol ranged from 90-204, 35-65 and 232-259 ng/g in cheetah (Jurke *et al*., 1997), tiger (Dembiec *et al*., 2004) and jaguar (Morato *et al*., 2004), respectively. Faecal concentration of cortisol was 140 and 51 ng/g in male and female cats, respectively (Graham and Brown, 1996). Data clearly demonstrate that level of cortisol differ according to species and sexes. Thus, it would be desirable to generate species specific baseline data that represent specific physiological status and husbandry protocol.

Irrespective of treatments, level of cortisol reported herein were higher than that reported earlier in free ranging felids. Faecal concentration of cortisol in zoo-housed cheetah was higher than their wild counterparts (Terio *et al.,* 2004). Reduced concentration of cortisol was associated with alleviation of stress (Morgan and Tromberg, 2009). The welfare of the zoo-housed leopards is compromised for many reasons such as restricted movement, compelled human proximity, reduced feeding opportunity and improper social structure (reviewed by Morgan and Tromberg, 2007). Thus, it would be desirable to explore ways through which it may be possible to alleviate stress and improve welfare. In this experiment, replacement of buffalo meat with chicken resulted in decreased faecal concentration of cortisol. In wild, leopard is a ubiquitous predator that feeds on several species of mammals and birds (Andheria *et al.,* 2007). However, in captivity they are fed solely on buffalo meat. Feeding of monotonous diet in confinements may adversely influence animal welfare (Catanese *et al*., 2013). Gradual replacement of BM with chicken provided an alternate feed source that added some variety to the monotonous buffalo meat diet fed to the leopards that might have reduced boredom and increased the welfare of the animals. Additionally, replacement buffalo meat with chicken increased supply of carotenoids that acts as a potent antioxidant capable of reducing oxidative stress. All these factors might have contributed to the reduced faecal concentration of cortisol in leopards fed diet containing chicken. Our results are similar to those reported earlier which indicate that supplementation of exogenous antioxidants can reduce circulatory glucocorticoids, alleviate stress and improve welfare (Peters *et al*., 2001).

Irrespective of the dietary treatments, concentration of cortisol was higher in serum than feces. Cortisol may be degraded in the gut by bacterial enzymes. An anaerobic bacteria isolated from human faeces caused 21-dehydroxylation of glucocorticoid metabolites (Winter *et al*., 1979). Even though, feces is the predominant route of excretion of glucocorticoid metabolites (GCM) in felids, only 86% of the GCM were recovered in feces (Graham and Brown, 1996). In male cats, only 78% of the GCM were excreted in feces. Thus, all the corticoids may not be recovered in the feces. There was a dose-dependent decline in concentration of cortisol with increase dietary proportion of chicken and was positively related with carotenoids intake. It was possible to measure this stress reducing response by measuring cortisol in both serum and faecal sample. Additionally, we observed a positive relationship between serum and faecal concentration of cortisol. Thus, the stress reducing response of dietary supplementation of antioxidants in zoo-housed Indian leopards can be studied by using non-invasive method as well. A comparison further revealed that within sample variation was higher and R^2 was lower while cortisol was measured in serum samples as compared to faecal samples. Thus, it would be desirable to measure cortisol in feces rather than in serum to monitor stress in zoo-housed Indian leopards and to study the response of dietary supplementation of antioxidants in ameliorating stress in this species.

CONCLUSION

Assay of faecal samples indicated that replacement of buffalo meat with chicken at 20% in the diet would increase the antioxidant activity and reduce oxidative stress in captive leopards. Regression analysis revealed positive relationship between serum and faecal sample assay with respect to both TAA and cortisol. Thus, faecal samples could be used to monitor cortisol and antioxidant status in captive Indian leopards.

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